CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*

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Engulfment of apoptotic cells in *Caenorhabditis elegans* is controlled by two partially redundant pathways. Mutations in genes in one of these pathways, defined by the genes *ced-2*, *ced-5* and *ced-10*, result in defects both in the engulfment of dying cells and in the migrations of the two distal tip cells of the developing gonad. Here we find that *ced-2* and *ced-10* encode proteins similar to the human adaptor protein CrkII and the human GTPase Rac, respectively. Together with the previous observation that *ced-5* encodes a protein similar to human DOCK180, our findings define a signalling pathway that controls phagocytosis and cell migration. We provide evidence that CED-2 and CED-10 function in engulfing rather than dying cells to control the phagocytosis of cell corpses, that CED-2 and CED-5 physically interact, and that *ced-10* probably functions downstream of *ced-2* and *ced-5*. We propose that CED-2/CrkII and CED-5/DOCK180 function to activate CED-10/Rac in a GTPase signalling pathway that controls the polarized extension of cell surfaces.

Cells that die by programmed cell death (apoptosis) are rapidly removed by phagocytosis[1]. A molecular pathway that controls the execution of programmed cell death has been elucidated in *C. elegans*[2], and a similar pathway was subsequently found to regulate the execution of cell death in mammalian cells[3]. In contrast, the mechanisms that cause the rapid removal of cell corpses by phagocytosis have remained largely unknown.

In *C. elegans*, the six genes known to control cell-corpse removal[4,5] have been grouped into two classes, *ced-2*, *ced-5* and *ced-10*, and *ced-1*, *ced-6* and *ced-7*, on the basis both of genetic interactions[5] and, more recently, of a defect in the migrations of the two gonadal distal tip cells (DTCs) in mutants of the *ced-2, ced-5* and *ced-10* class[6]. *ced-6* encodes a possible adaptor-like protein[7] and *ced-7* an ABC-type transporter protein[8]. CED-5 is similar to human DOCK180 and *Drosophila melanogaster* Myoblast City[6], both of which have been implicated in regulating cell morphology[9,10]. In this work, we aimed to elucidate the pathway in which CED-5 acts, by characterizing *ced-2* and *ced-10*.

Results

***ced-2*, *ced-5* and *ced-10* mutants are defective in DTC pathfinding.** To understand better the roles of *ced-2*, *-5* and *-10* in the control of cell migration, we characterized the DTC-migration paths in mutant animals. The DTCs migrate to direct the development of the *C. elegans* bilobed gonad[11]. In *ced-2*, *-5* and *-10* mutants, the DTCs were more frequently defective in pathfinding (that is, in migrating along a wild-type path) than in movement (initiating and maintaining migration) (Table 1). The most frequent gonadal defect in *ced-2*, *-5* and *-10* mutants was an abnormal extra turn of the gonad arm, resulting from a reversal in the direction of DTC migration along the dorsal musculature (Fig. 1; data not shown). *ced-10* mutants exhibited more penetrant cell-movement defects than did *ced-2* or *ced-5* mutants. That the penetrances of the DTC-pathfinding defects were greater than those of the movement defects indicates that *ced-2*, *ced-5* and *ced-10* may primarily control the polarization, rather than the generation, of DTC-surface extensions. Similarly, the defects in cell-corpse engulfment in *ced-2*, *-5* and *-10* mutants might be caused by abnormalities in polarized cell-surface extensions of the engulfing cells.

***ced-2* encodes a protein similar to CrkII.** To characterize *ced-2*, we determined its molecular identity. *ced-2* had previously been mapped on the left arm of chromosome IV (ref. 5) (Fig. 2a). We observed the presence of a gene, Y41D4A_3457.b (Fig. 2a), in the physical map of this region[12] predicted to encode a product similar to CrkII. CrkII, produced as the predominant alternatively spliced isoform of the *Crk* locus, is an adaptor protein consisting of an SH2 (Src-homology-2) domain followed by two carboxy-terminal SH3 (Src-homology-3) domains[13]. The characterization of the viral oncoprotein v-Crk was instrumental in the discovery and understanding of signal-transduction adaptor proteins[14]. CrkII localizes to focal adhesions with $p130^{Cas}$ and DOCK180, and has been implicated in cell migration[15,16]. DOCK180, a protein similar to CED-5 (ref. 6), was isolated on the basis of its physical interaction with Crk[9].

Given the interaction between DOCK180 and CrkII and the possible role of CrkII in cell migration, we sought to determine whether Y41D4A_3457.b is *ced-2*. We determined the sequence of a

Table 1 *ced-2* and *ced-10* mutants have defects in phagocytosis and DTC migration

		Gonadal arms with migration abnormality*	
Genotype	**Persistent corpses†**	**Length‡**	**Morphology§**
Wild-type	0.0 ± 0.0	0%	2%
ced-2(n3238)	14.8 ± 3.5	0%	21%
ced-2(e1752)	16.3 ± 3.7	1%	39%
ced-2(n1994)	20.0 ± 3.3	0%	38%
ced-5(n1812)	31.0 ± 3.6	6%	51%
ced-10(n1993)	18.2 ± 3.8	15%	35%
ced-10(n3246)	26.0 ± 3.9	12%	34%

* The percentage of gonadal arms abnormal for DTC migration was determined from 50 anterior and 50 posterior arms using Nomarski microscopy.
† Heads of young L1 larvae at the four-cell gonad stage were scored by Nomarski microscopy for persistent cell corpses. Data are means ± s.d. from at least 50 animals.
‡ Gonadal arms that were short or grossly disorganized with germ cells dispersed inside the body cavity were categorized as defective in length and interpreted as abnormal in DTC movements.
§ The percentage of gonadal arms, of those in which DTCs migrated, with inappropriate turns, twists, or positions categorized as abnormal in morphology and interpreted as abnormal in DTC pathfinding.

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Y41D4A_3457.b complementary DNA, yk435b1, and determined that the Y41D4A_3457.b gene consists of three exons (Fig. 2a) and encodes a predicted product with 34% identity to human CrkII (Fig. 2b). We used RNA-mediated interference (RNAi)[17] of Y41D4A_3457.b to determine its loss-of-function phenotype. RNAi resulted in defects in the migration of the DTCs that were similar to the defects in *ced-2* mutant animals, but did not cause defects in cell-corpse engulfment (data not shown). DTC migration requires zygotic *ced-2* function, whereas cell-corpse engulfment requires maternal *ced-2* function (data not shown), indicating that RNAi may affect only the zygotic functions of this locus. We determined the sequence of the genomic locus of Y41D4A_3457.b in *ced-2* mutant animals and identified mutations, all of which are predicted to result in truncated CED-2 proteins, in each of the three existing alleles (Fig. 2b). We rescued both the phagocytosis (see below and Table 2) and DTC-migration (Table 3) defects of *ced-2* mutant animals by expression of the Y41D4A_3457.b cDNA using *C. elegans* heat-shock promoters. Together, these findings establish that Y41D4A_3457.b is *ced-2*.

Mutant mice containing an incomplete deletion of the *Crk* locus have been found to be viable with no obvious defects[18]. These mice express a *Crk* transcript predicted to make a protein that lacks only the second SH3 domain and also presumably express the *CrkL* gene, the product of which is similar to CrkII (ref. 19). The first SH3 domain, which is necessary[15] and sufficient[9] for binding to DOCK180, is left intact by this mutation. The role of Crk proteins during animal development and the pathways in which they normally function have thus been largely unresolved, because, to our knowledge, no other mutations in any *Crk* gene have been described. Our results show that *ced-2*/*CrkII* controls cell migration and the phagocytosis of apoptotic cells *in vivo*.

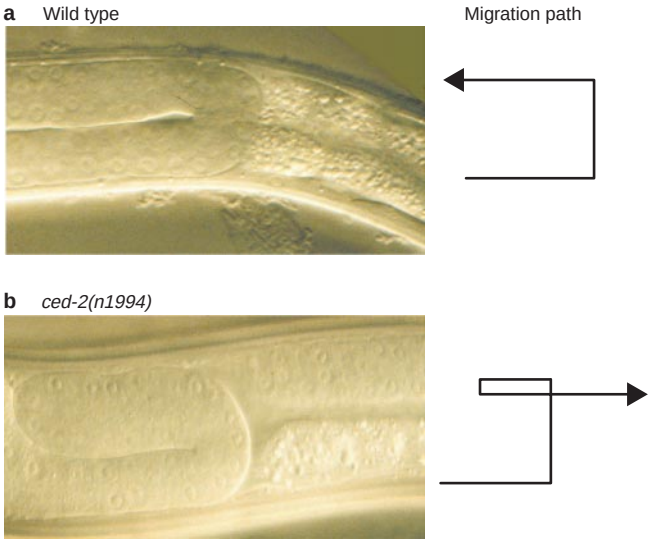

Figure 1 **The distal tip cell (DTC) reverses its direction of migration in a *ced-2* mutant. a**, Wild-type DTC. The posterior DTC migrates posteriorly along the ventral side, then dorsally and finally anteriorly along the dorsal side. **b**, *ced-2(n1994)* DTC. The posterior DTC migrated posteriorly along the ventral side, then dorsally and anteriorly along the dorsal side; it then reversed direction and migrated posteriorly along the dorsal side. Left, Nomarksi photomicrographs; right, diagrams of DTC migratory paths. Arrowheads indicate direction of DTC migration. Anterior is to the left, and ventral is down.

CED-2/CrkII and CED-5/DOCK180 interact physically. Our finding that loss of function of either *ced-2*/*CrkII* or *ced-5*/*DOCK180* results in equivalent defects provides genetic evidence in support of the idea that the previously revealed physical interactions between CrkII and DOCK180 (ref. 9) occur *in vivo*. We found that CED-2 can interact physically with CED-5. Specifically, the [^{35}S]methionine-labelled C-terminal region of CED-5 (ΔN-CED-5), which contains a candidate SH3-binding proline-rich region[6], associated with glutathione-*S*-transferase (GST)-tagged CED-2 (Fig. 3). CED-2 and CED-5 also interacted in the yeast two-hybrid system (data not shown).

***ced-10* encodes a protein similar to Rac.** We also determined the molecular identity of the *ced-10* gene. We genetically mapped *ced-10* to the left arm of chromosome IV, near *unc-17* and *unc-33* (see Methods) (Fig. 2a). We studied the physical map of the region containing *unc-17* and *unc-33* (refs 20, 21) and noted the presence of the *rac-1* gene (Fig. 2a), previously identified by hybridization to a human Rac cDNA; the RAC-1 protein is 84% identical to human Rac and is a functional GTPase[22] (Fig. 2c). Rac, Rho and Cdc42 define a subgroup of the Ras-GTPase superfamily involved in the control of cytoskeletal organization and cell extensions[23] and in phagocytosis mediated by immunoglobulin and complement receptors[24,25]. Interactions between DOCK180 and Rac have been observed[26,27].

Given the similar map positions of *ced-10* and *rac-1* and the possible role of Rac in controlling cell polarity and migration[23], we sought to determine whether *rac-1* is *ced-10*. *rac-1* may exist as the downstream gene in an operon. The gene immediately 5′ of *rac-1* encodes a predicted protein of unknown function in *C. elegans* similar to mammalian TSG101, a tumour-susceptibility protein[28]. A northern blot probed with a *rac-1* cDNA identified transcripts of about 1.7 and 0.9 kilobases, consistent with *rac-1* being a member of an operon[22]. Like many *C. elegans* messenger RNAs, *rac-1* transcripts can carry *trans*-spliced leader (SL) sequences (see Methods)[29]. *rac-1* transcripts can be either SL1 or SL2 *trans*-spliced, consistent with the hypothesis that *rac-1* is a downstream gene in an operon, as messages from downstream genes in *C. elegans* operons often carry SL2 leader sequences[29]. We used RNAi to determine the phenotype produced by loss of *rac-1* function.

Table 2 Expression of *ced-2* and *ced-10* outside dying cells rescued engulfment defects

			Number of persistent corpses*				
Genotype	**Transgene**	**Heat shock**	**L1**	**L2**	**L3**	**L4**	**Germ line†**
ced-2(e1752)	P_{hsp} *gfp*	+	18.6 ± 3.1	15.4 ± 3.1	13.2 ± 3.8	8.9 ± 2.9	15.9 ± 6.2
	P_{hsp} *ced-2*	–	17.7 ± 4.5	14.2 ± 5.1	12.0 ± 3.8	8.9 ± 2.8	16.3 ± 7.5
	P_{hsp} *ced-2*	+	3.1 ± 1.5	2.7 ± 1.6	1.9 ± 1.3	1.2 ± 1.1	2.2 ± 2.9
ced-10(n3246)	P_{hsp} *gfp*	+	27.3 ± 4.2	25.5 ± 3.6	22.3 ± 3.8	17.5 ± 3.9	11.7 ± 4.9
	P_{hsp} *ced-10*	–	25.8 ± 4.3	24.4 ± 3.9	20.3 ± 3.2	15.9 ± 3.3	14.5 ± 5.8
	P_{hsp} *ced-10*	+	4.0 ± 2.9	3.7 ± 2.5	2.4 ± 1.9	2.1 ± 1.4	1.8 ± 0.2

* The number of persistent corpses shown was determined from two independent transgenic lines for each specified transgene, P_{hsp} *gfp*, P_{hsp} *ced-2* and P_{hsp} *ced-10*. Data are means ± s.d. from at least 40 animals, at least 20 from each of the two independent lines, at each stage.

† In each animal only one gonadal arm, that on the side of the animal mounted upwards on a slide, was scored using Nomarski optics for persistent germline corpses. Data are means ± s.d. from at least 40 animals, at least 20 from each line, for single gonadal arms.

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RNAi resulted in defects in both cell-corpse engulfment and DTC migration (data not shown).

We determined the sequence of the genomic locus of *rac-1* in *ced-10* mutant animals and identified mutations in each of the two existing alleles. *ced-10*(*n1993*) animals have a Val 190 to Gly change in the penultimate amino acid of the predicted protein, affecting a possible CaaX prenylation site (where 'a' represents an aliphatic amino acid and 'X' typically represents leucine within prenylation sites that are targets of type I geranylgeranyltransferases[30]). Prenylation is a conserved mechanism for the membrane targeting of Ras-like GTPases[30]. This penultimate amino acid can be important for the ability of a protein to be prenylated (for example, glycine at this position in a tetrapeptide was 100-fold less effective than valine in inhibition of farnesyltransferase[31]). *ced-10*(*n3246*) animals have a Gly 60 to Arg mutation in a conserved DTAG motif implicated in the binding of the γ-phosphoryl group of GTP. This glycine residue is highly conserved among all GTPases and may be necessary for GTP-binding-induced conformational changes[32]. Judged on the basis of known GTPase structures and functions, both mutations probably result in reduced signalling activity. We rescued both the phagocytosis (see below and Table 2) and DTC-migration (Table 3) defects of *ced-10* mutant animals by expression of a *rac-1* cDNA using *C. elegans* heat-shock promoters. Together, these findings establish that *rac-1* is *ced-10*.

Mutant mice that lack Rac1 function have been reported to be inviable with pleiotropic defects proposed to result from abnormalities in cell adhesion[33]. The pathways within which Rac1 normally acts to control specific aspects of animal development remain largely unknown. Our results show that CED-10/Rac controls controls cell migration and the phagocytosis of apoptotic cells *in vivo* in a signal-transduction pathway with CED-2/CrkII and CED-5/DOCK180.

***ced-2* and *ced-10* probably function within engulfing cells.** To determine whether *ced-2* and *ced-10* function within dying or engulfing cells to control the phagocytosis of cell corpses, we performed ectopic expression experiments. Specifically, we expressed *ced-2* in *ced-2* mutants and *ced-10* in *ced-10* mutants under the control of *C. elegans* heat-shock promoters, first long after cells had died and failed to be engulfed, and second during the production of germline cell corpses. Both expression protocols allowed the rapid phagocytosis of unengulfed cell corpses (Table 2). Somatic cell corpses were unlikely to have expressed *ced-2* or *ced-10* long after the cells had died, because no expression of the green fluorescent protein (GFP) from *C. elegans* heat-shock promoters

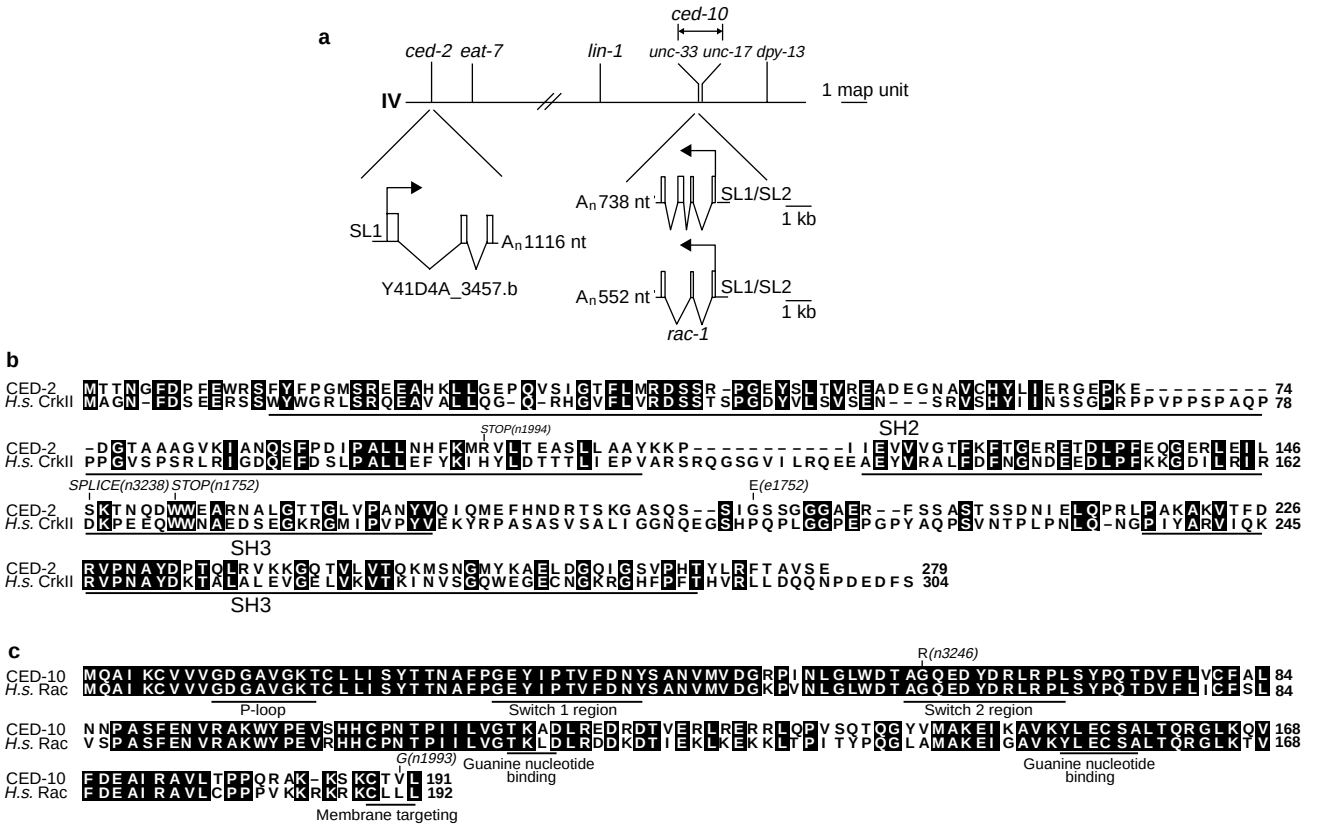

Figure 2 ***ced-2* and *ced-10* encode proteins similar to human CrkII and Rac, respectively. a**, Top, *ced-2* and *ced-10* both map to the left arm of chromosome IV. Bottom, structures of the genomic loci of Y41D4A_3457.b (*ced-2*; left) and *rac-1* (*ced-10*; right) were inferred from cDNA sequences. The 5′ and 3′ ends of the mRNAs were determined by RACE (see Methods). 5′ ends of *ced-2* mRNAs contained SL1 *trans*-spliced leaders, commonly found in *C. elegans* transcripts[39], and 5′ ends of *ced-10* mRNAs were either SL1 or SL2 *trans*-spliced. A 552-nucleotide (nt) isoform of *rac-1* mRNA, identified (Y. Kohara, personal communication) from an expressed sequence tag from a cDNA library and predicted to produce a RAC-1 protein lacking a region involved in nucleotide binding, is shown below the 738-nt mRNA. **b**, Alignment of the CED-2 amino-acid sequence with that of human CrkII. Identical amino acids are boxed and positions of mutations are labelled. *ced-2(n1994)* is a C-to-T mutation, resulting in an R102opal nonsense allele; *ced-2(n3238)* is a G-to-A splice-site mutation; and *ced-2(e1752)* animals have two different G-to-A mutations within the *ced-2* open reading frame, resulting in W153opal nonsense and G192E missense changes. **c**, Alignment of the amino-acid sequences of *C. elegans* CED-10 and human Rac. Identical amino acids are boxed and positions of mutations are labelled. *ced-10(n3246)* is a G-to-A missense mutation resulting in a G60R substitution, and *ced-10(n1993)* is a T-to-G missense mutation resulting in a V190G substitution. The P-loop region interacts with the phosphoryl groups of GDP and GTP, and the switch regions change conformation upon GDP/GTP exchange[27]. kb, kilobase.

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is detectable in cells that have died[6]; in other words, dead cells cannot synthesize new mRNAs or proteins. Other somatic cells, including engulfing cells, do express GFP, and thus presumably *ced-2* and *ced-10*, from these promoters at these times. Similarly, germline corpses were unlikely to have expressed *ced-2* or *ced-10*, as germline gene expression typically does not occur from transgenes in extrachromosomal arrays (A. Fire, personal communication), and as the germ line does not express detectable levels of GFP from these heat-shock promoters[6]. Germline corpses are engulfed by the somatic sheath cells[34], which do express GFP and thus presumably *ced-2* or *ced-10* from these heat-shock promoters[6]. Taken together, these findings indicate that the expression of *ced-2* and *ced-10* in cells other than dying cells is sufficient to allow cell-corpse engulfment. Therefore, we suggest that CED-2 and CED-10 are likely to act within engulfing rather than dying cells to control the phagocytosis of cell corpses.

***ced-10* may function downstream of *ced-2* and *ced-5* in corpse engulfment.** To understand better the genetic pathway in which *ced-2*, *ced-5* and *ced-10* function, we performed bypass experiments (Table 3). In these experiments, *ced-2* overexpression did not rescue the phagocytosis or migration defects of *ced-5* or *ced-10* mutants, and *ced-5* overexpression did not rescue the phagocytosis or migration defects of *ced-2* or *ced-10* mutants. In contrast, *ced-10* overexpression did bypass the functional requirement for *ced-2* or *ced-5* in cell-corpse phagocytosis (Table 3). The phagocytosis and DTC-migration defects of *ced-2*(*n1994*) mutants, which are predicted to produce CED-2 protein that is truncated before both SH3 domains (Fig. 2b), were both rescued by *ced-10* overexpression. The phagocytosis defect of *ced-5*(*n1812*) mutants, which are predicted to be null for *ced-5* function[6], was similarly rescued

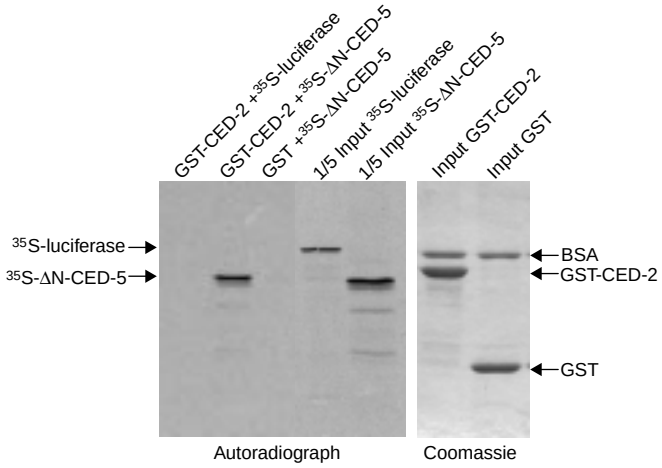

Figure 3 **CED-2 and CED-5 interact.** Left, autoradiograph. GST–CED-2 did not interact with ^{35}S-labelled luciferase (lane 1) but did interact with the ^{35}S-labelled C-terminal 374 amino acids (amino acids 1,407–1,781) of CED-5 (ΔN-CED-5) (lane 2). ^{35}S-labelled ΔN-CED-5 did not interact with GST alone (lane 3). Lanes 4, 5: 20% of the amount of ^{35}S-labelled proteins used in binding reactions. Right, Coomassie blue staining. Roughly equal amounts of GST–CED-2 and the control GST protein were used in the binding reactions. See Methods.

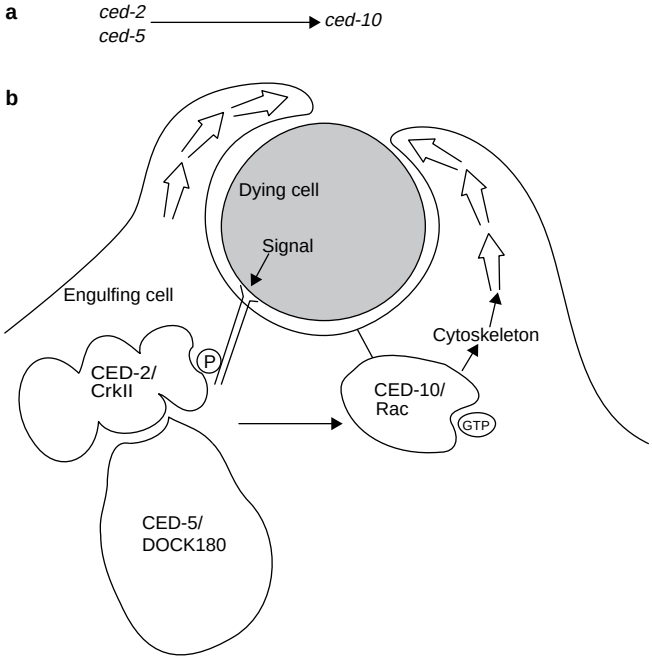

Figure 4 **Model for the control of phagocytosis in programmed cell death by *ced-2*, *ced-5* and *ced-10*. a**, *ced-10* acts downstream of *ced-2* and *ced-5* in a genetic pathway. **b**, CED-2/CrkII and CED-5/DOCK180 transduce apoptotic signals to activate the GTPase CED-10/Rac. Circled 'P', phosphorylation.

Table 3 Overexpression of *ced-10* bypassed *ced-2* and *ced-5* mutant defects in cell-corpse engulfment

		Number of persistent corpses*			Percentage of animals with DTC-migration defects†		
Transgene‡	**Heat shock**	***ced-2(n1994)***	***ced-5(n1812)***	***ced-10(n3246)***	***ced-2(n1994)***	***ced-5(n1812)***	***ced-10(n3246)***
P_{hsp} *gfp*	+	18.7 ± 5.0	31.7 ± 5.1	26.7 ± 4.8	34 ± 5%	53 ± 9%	39 ± 10%
P_{hsp} *ced-2*	–	17.9 ± 5.1	31.0 ± 4.6	28.3 ± 3.7	32 ± 5%	53 ± 5%	43 ± 4%
P_{hsp} *ced-2*	+	1.3 ± 1.0	32.7 ± 4.7	27.9 ± 3.2	3 ± 1%	55 ± 3%	45 ± 2%
P_{hsp} *ced-5*	–	21.1 ± 3.5	32.3 ± 5.9	26.8 ± 4.4	37 ± 3%	56 ± 4%	49 ± 6%
P_{hsp} *ced-5*	+	21.6 ± 5.4	1.0 ± 1.6	27.5 ± 4.2	33 ± 6%	3 ± 1%	47 ± 11%
P_{hsp} *ced-10*	–	19.5 ± 3.2	31.2 ± 4.3	28.0 ± 3.5	27 ± 2%	52 ± 4%	38 ± 0%
P_{hsp} *ced-10*	+	1.0 ± 1.2	2.9 ± 1.4	0.3 ± 0.4	7 ± 1%	51 ± 12%	11 ± 6%

* The numbers of persistent corpses were determined from L1 and L2 larvae heads using Nomarski optics. Data are means ± s.d. from at least 20 animals.

† The percentages of animals with DTC-migration defects were inferred using Nomarski optics from the gonad morphology of young adults. To minimize subjectivity, only animals with severe defects, including ectopic turns and disorganization, were counted as abnormal. Data are average percentages ± s.d. from at least two independent experiments. At least 50 animals of each line were scored in each experiment.

‡ At least two independent extrachromosomal arrays for each of the transgenes, P_{hsp} *ced-2*, P_{hsp} *ced-5*, and P_{hsp} *ced-10*, were found to behave similarly. Overexpression of either *ced-2* or *ced-5* did not interfere with cell-corpse engulfment in wild-type animals (data not shown).

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by *ced-10* overexpression. *ced-10* overexpression in *ced-5*(*n1812*) animals resulted in changes in the observed patterns of DTC migration (data not shown) but was not efficient in restoring normal patterns. Perhaps this incomplete rescue reflects a need for a greater level of CED-5 function in DTC migration than in phagocytosis. We suggest that *ced-10* functions downstream of *ced-2* and *ced-5* in a genetic pathway controlling cell-corpse engulfment (Fig. 4a), as overexpression of neither *ced-2* nor *ced-5* bypassed the requirement for other pathway components, and overexpression of *ced-10* bypassed the requirement for *ced-2* and *ced-5*.

Discussion

Cell migration and the engulfment of apoptotic cells are fundamental biological processes involving membrane polarization and cytoskeletal dynamics[35,36]. Here we have described our discovery in *C. elegans* of a GTPase signalling pathway that acts to control both cell migration and the engulfment of apoptotic cells.

Previous biochemical studies indicated that Crk and DOCK180 can interact physically[9], but whether or not this interaction is physiologically important was unknown. Similarly, no developmental role had been defined for any *Crk* gene. In *Drosophila*, mutations in *myoblast city* (*mbc*) were isolated as suppressors of a rough-eye phenotype induced by ectopic expression of *rac1* (ref. 27). That ectopically expressed *Drosophila rac1* required *mbc* activity for its function indicates either that *rac1* acts through *mbc* or that *mbc* is needed to activate *rac1*. However, loss-of-function *Drosophila rac1* mutations have not been described, so it has not been established whether *rac1* and *mbc* normally interact functionally in flies. Similarly, dominant-negative mammalian Rac1 can suppress DOCK180-induced membrane spreading[26], but no loss-of-function data exist to establish whether Rac1 and DOCK180 interact physiologically. Other biochemical and cell biological experiments have also indicated, but not established, that DOCK180 may act in a pathway with, and upstream of, Rac1 (refs 26, 27).

Our results establish that CED-2/CrkII, CED-10/Rac and CED-5/DOCK180/Mbc function together *in vivo* in a pathway that controls both cell migration and cell-corpse phagocytosis. Furthermore, the fact that overproduction of CED-10 can bypass the requirement for CED-5 in a process that CED-10 normally regulates, namely cell-corpse phagocytosis, strongly favours a model in which CED-5/DOCK180/Mbc acts through CED-10/Rac. We propose that the CED-2/CrkII adaptor protein localizes CED-5/DOCK180 to the plasma membrane, leading to the activation of the CED-10/Rac GTPase within engulfing and migrating cells (Fig. 4b), and that such activation regulates the polarity of cytoskeletal extensions in response to apoptotic or migrational cues. Given the molecular and functional conservation between CED-2, CED-5 and CED-10 and their human counterparts, we suggest that these proteins function in an evolutionarily conserved signalling pathway that is likely to have similar developmental functions in most, if not all, metazoans. □

Methods

cDNA structure, RNAi, mapping and allele sequencing.

The *ced-2* cDNA plasmid pPR23 was excised from the phage clone yk135b1 (from Y. Kohara). *ced-10* cDNA, pPR37, was isolated by the polymerase chain reaction (PCR) from *C. elegans* mixed-stage cDNA and cloned into pGEM-2T (Promega). To determine the 5′ ends of the *ced-2* and *ced-10* messages, we used a 5′-rapid amplification of cloned ends (RACE) system (Gibco). In addition to the SL1 and SL2 leaders, we recovered two variants of the SL2 splice leader, with sequences GGTTTTAACCCAGTTAACCAAG and GGTTTTAACCCAAGTTAAGCAG, on *ced-10* messages. To determine the 3′ ends of *ced-10* mRNAs, we used a 3′-RACE system (Gibco). pPR23 and pPR37 were used as templates for RNA production, and RNAi was performed as described[17]. Allele sequences from PCR products of genomic regions and DNA sequences of cDNAs were determined using an automated ABI 373A DNA sequencer (Applied Biosystems). We were unable to obtain recombinants between *ced-10*(*n1993*) and *unc-33*(*e204*) among 15 recombinants in the region between *lin-1* and *unc-33*, or between *ced-10*(*n1993*) and *unc-17*(*e113*) among 39 recombinants in the region between *unc-17* and *dpy-13*. The alleles *ced-2*(*e1752*), *ced-2*(*n1994*), and *ced-10*(*n1993*) have been described previously[4,5]. *ced-2*(*n3238*) and *ced-10*(*n3246*) were isolated and provided by Z. Zhou.

Binding experiments.

To obtain GST–CED-2, we cloned *ced-2* cDNA into the vector pGEX-2T (Pharmacia), expressed this vector in BL21(DE3) cells, and purified the protein as recommended (Pharmacia). To obtain [^{35}S]ΔN-CED-5, we cloned a *ced-5* cDNA fragment, encoding amino acids 1,407–1,781, into pCITE-4a(+) (Novagen), and transcribed and translated this fragment as recommended (TNT Coupled Reticulocyte Lysate System, Promega). GST–CED-2, bound to glutathione–Sepharose-4B (Pharmacia), and [^{35}S]ΔN-CED-5 were incubated for 60 min at 4 °C in 100 µl binding buffer (150 mM NaCl, 0.1% Tween-20, 25 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 0.1 mM EDTA). Protein complexes were washed three times with 1 ml washing buffer (300 mM NaCl, 0.1% Tween-20, 25 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 0.1 mM EDTA) with 0.25% BSA and once with washing buffer without BSA, and analysed by SDS–PAGE (10%) and autoradiography.

Germline transformation.

P_{hsp} *ced-2* was made by ligating a *Kpn*I (blunted with T4 DNA polymerase)/*Spe*I pPR23 fragment to *Nhe*I/*Eco*RV-digested pPD49.78 and pPD49.83 (from A. Fire). P_{hsp} *ced-10* was made by ligating a *Spe*I/*Nco*I pPR37 fragment to *Nhe*I/*Nco*I-digested pPD49.78 and pPD49.83. P_{hsp} *gfp* was made as described[6]. Standard methods[37] were used to create *ced-2*(*e1752*) transgenic lines carrying P_{hsp} *ced-2* or P_{hsp} *gfp*. *ced-2(e1752); unc-76(e911)* animals were injected with P_{hsp} *ced-2* or P_{hsp} *gfp* along with a P_{sgl-5} *gfp* construct (A. Chisholm and H.R.H., unpublished observations), all at 50 mg ml^{-1}, with an *unc-76*-rescuing construct, p76-16B (ref. 38), at 75 mg ml^{-1}. Transgenic animals were non-Unc and GFP-positive. *ced-10*(*n3246*) animals were injected with either P_{hsp} *ced-10* or P_{hsp} *gfp* at 50 mg ml^{-1} along with P_{myo-3} *gfp* (from A. Fire) at 35 mg ml^{-1}. Transgenic animals were GFP-positive.

Heat-shock experiments.

To test for rescue of unengulfed somatic corpses in *ced-2* and *ced-10* mutant animals, we shifted transgenic animals from 15 °C to 33 °C for 1 h and allowed animals to recover at 20 °C for 3–6 h before scoring. To test for rescue of unengulfed germline corpses we shifted L4 transgenic hermaphrodites from 20 °C to 33 °C for 1 h and allowed animals to recover at 20 °C. These animals were treated similarly every 12 h, and we scored germline corpses 44–48 h after the initial heat shock. For bypass experiments, we crossed rescuing lines carrying tp76-16B into *unc-76(e911)* animals and replaced the chromosomes carrying *ced* mutations with wild-type chromosomes. These lines were then crossed with *ced-2(n1994); unc-76(e911)*, *ced-5(n2812); unc-76(e911)*, or *ced-10(n3246); unc-76(e911)* mutant animals. We re-isolated rescuing lines to confirm that all transgenes were functional after crossing. P_{hsp} *ced-5* lines have been described previously[6]. To test for bypass of cell-corpse phagocytosis, we heat-shocked mixed-stage animals twice at 33 °C for 1 h, with a 10-h recovery period at 20 °C between heat shocks. Corpses in the heads of L1 to young L2 larvae were counted 10 h after the second heat shock. To rescue DTC-migration defects, we heat-shocked non-Unc L2 animals three times at 33 °C for 1 h, allowing animals to develop at 20 °C for 10–12 h between each treatment.

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Correspondence and requests for materials should be addressed to H.R.H. The nucleotide sequences of *ced-2* and *ced-10* have been deposited at GenBank under accession numbers AF226866 and AF226867, respectively.

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